Werewolf THERAPEUTICS

Shifting the Balance In Cytokine Therapeutics

SITC 2023

Optimal Antitumor Immunity is Triggered by WTX-124, a Clinical Stage Conditionally Activated INDUKINE™ Molecule that Releases Fully Potent IL-2 into the Tumor Microenvironment

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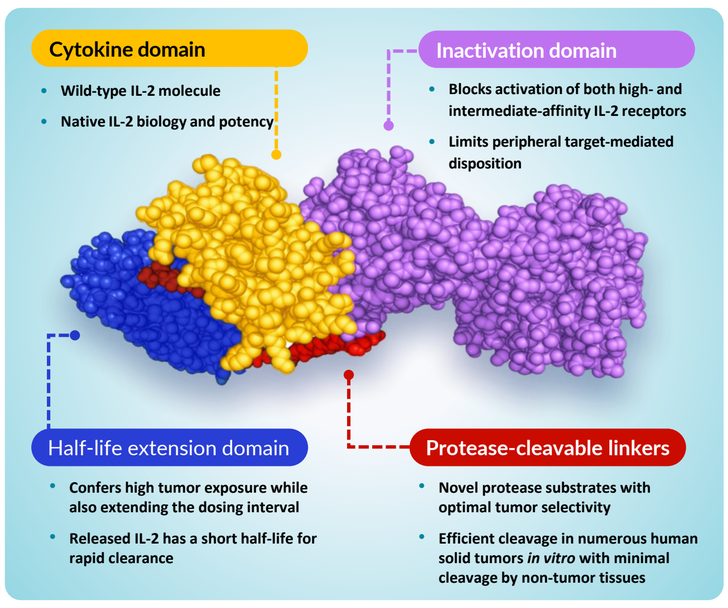
BACKGROUND

Interleukin-2 is a Promising Cytokine For Immunotherapy

Cytokine therapy could become a pillar of cancer immunotherapy given its potential to activate the immune system and promote antitumor activity. However, many cytokine therapies are limited in the clinic due to dose limiting toxicities associated with systemic administration. Recent approaches to improve upon IL-2 therapy have focused on developing attenuated forms of the cytokine (Non-Alpha formats) that are unable to bind to the high affinity IL-2 receptor, aiming to reduce the off-target toxicity and to minimize potential Treg suppressive effects[1]. However, Non-Alpha forms of IL-2 may also have reduced ability to activate the tumor specific T effector cells that drive anti-tumor immunity[2]. Indeed, modeling suggests that Non-Alpha molecules will need to be dosed at ~100 times the amount of a therapeutic delivering wildtype IL-2 to generate similar receptor occupancy in the tumor microenvironment[3]. To address the shortcomings of IL-2 therapy, we have developed WTX-124, a conditionally activated prodrug (INDUKINE™ molecule) that is designed to take advantage of the dysregulated protease milieu in the TME to deliver native IL-2 in a targeted fashion to tumor tissues after systemic administration. WTX-124 is better tolerated than half-life extended IL-2 in mice and generates robust anti-tumor immunity in several pre-clinical models[4]. To better understand the potential of IL-2 therapeutics carrying either wildtype or Non-Alpha IL-2 as a payload, we created an IL-2 INDUKINE molecule containing a Non-Alpha IL-2 moiety as reported in recent publications (IL-2V[1]) and compared its *in vitro* and *in vivo* activity to that of WTX-124.

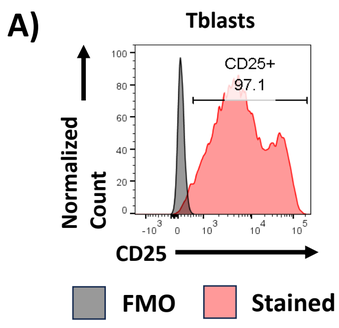
INDUKINE Design of WTX-124

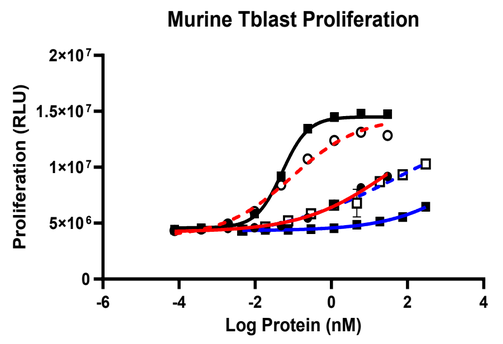
An IL-2 Prodrug for Cancer Therapy



Activated T Cells Respond More Efficiently to Wildtype IL-2

TCR Activation Induces High Affinity Receptor Expression *in vitro*



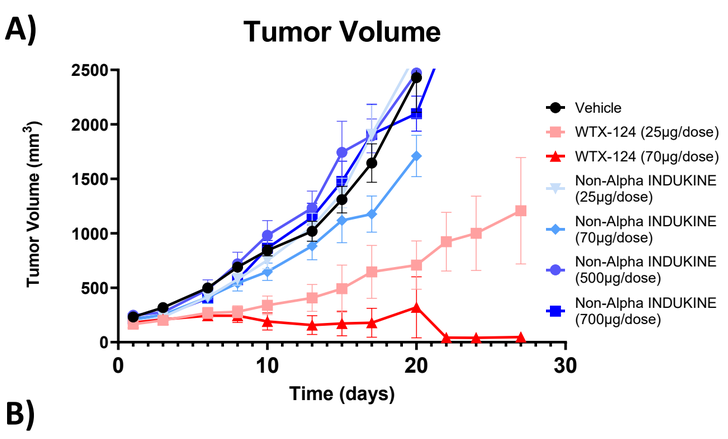


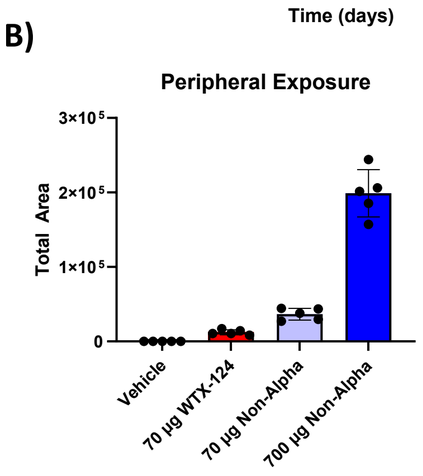
A) Splenocytes were stimulated with ConA. After 72 hours, Tblasts were stained by flow cytometry. Representative plot from CD8+ T cells is depicted. **B)** Tblasts were exposed to titrated amounts of rhIL-2 (WT), or with intact or cleaved (protease activated) INDUKINE molecules for 72 hours before proliferation was measured.

Molecule	EC_{50} (nM)
rhIL-2 (WT)	0.054
WTX-124: Intact	28.53
WTX-124: Cut	0.09
Non-Alpha Variant: Intact	~7690
Non-Alpha Variant: Cut	50.2

WTX-124 Generates Robust Anti-Tumor Activity

Non-Alpha INDUKINE Molecule is Substantially Less Potent

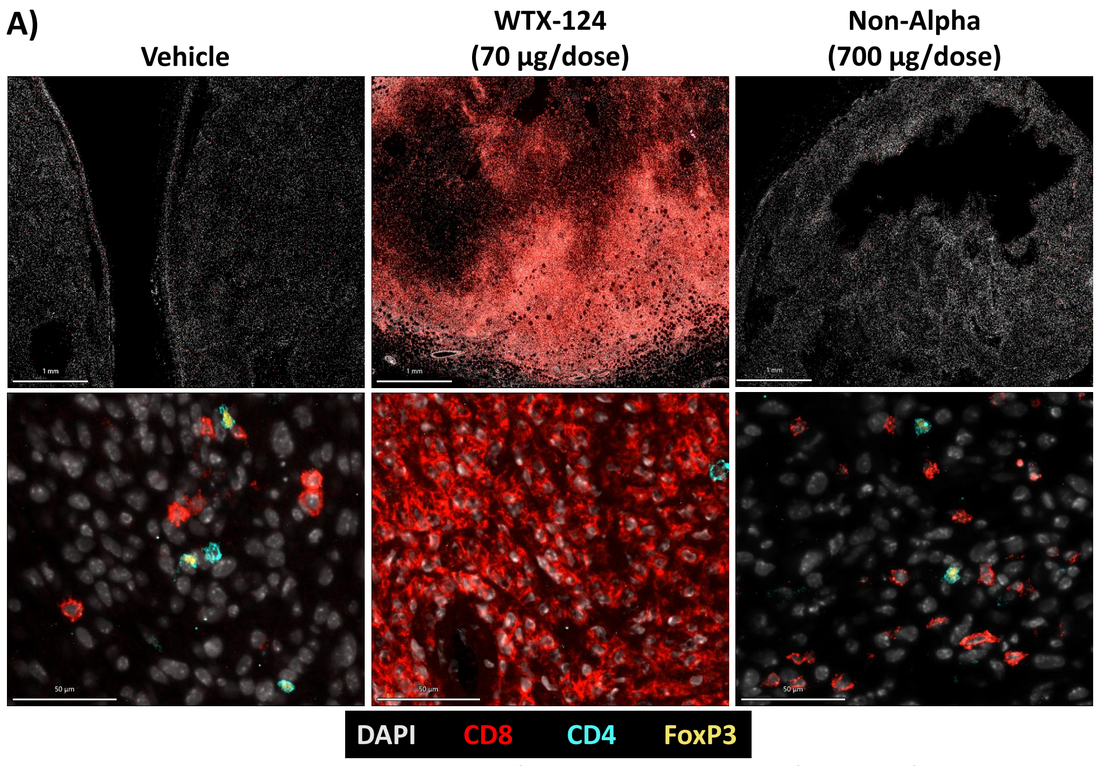


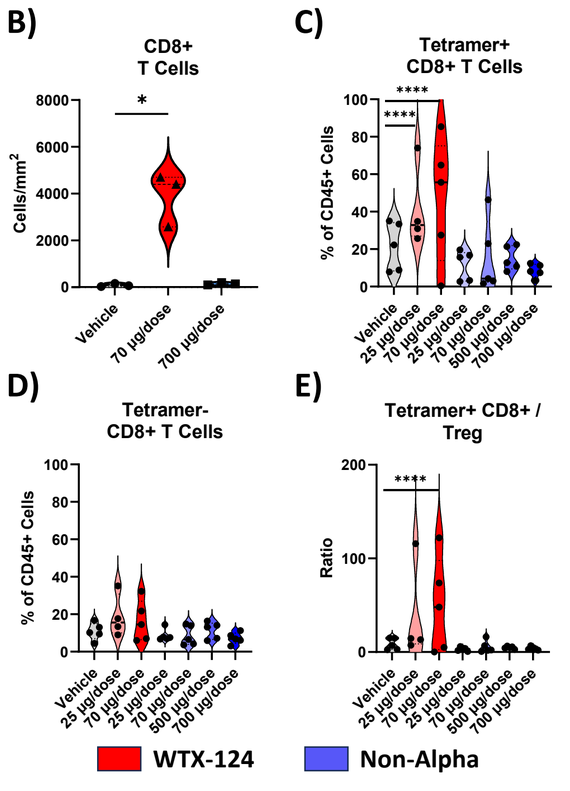


MC38 tumor bearing mice were dosed twice a week for a total of three doses with various doses of WTX-124, a variant IL-2 INDUKINE molecule containing a Non-Alpha IL-2 payload, or vehicle. Doses are specified in the figure. **A)** Tumor volume was measured twice a week. **B)** Murine plasma was harvested on Day 5, and total exposure of the individual therapeutic agents was measured by quantitative Western blot.

WTX-124 Preferentially Expands Tumor Specific CD8+ T Cells in the Tumor Microenvironment

Non-Alpha INDUKINE Molecule Fails to Expand Tetramer+ CD8+ T Cells

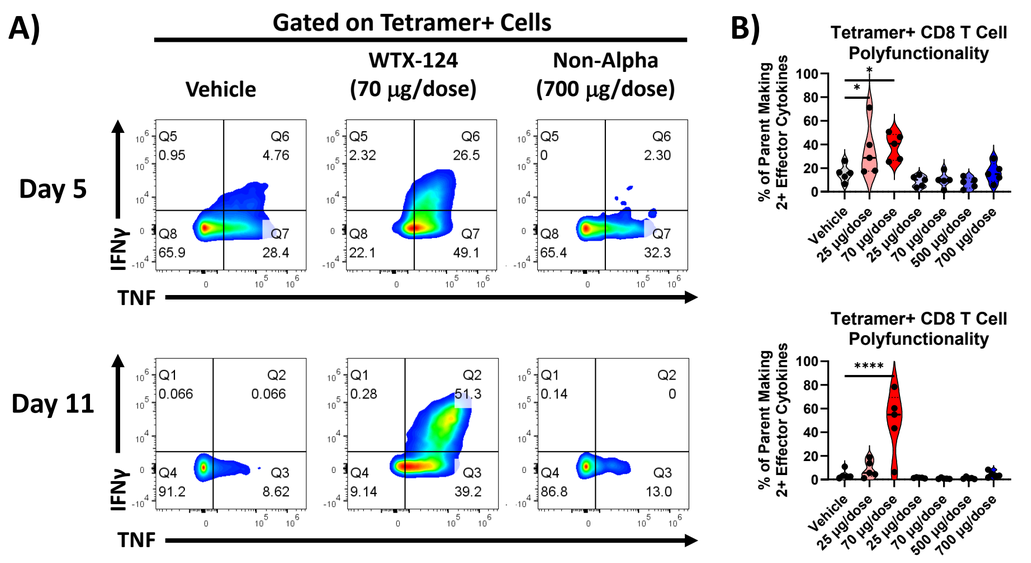




MC38 tumor bearing mice were dosed twice a week for two weeks with the specified doses of WTX-124, a variant IL-2 INDUKINE molecule containing a Non-Alpha IL-2 payload, or vehicle. Tumors were harvested on Day 5. Multiplexed immunofluorescence was performed on FFPE tumors, with **A)** representative images and **B)** quantification of tumor infiltration by CD8+ T cells. **C-E)** Tumors were dissociated and analyzed by flow cytometry. The frequency of **C)** tetramer+ or **D)** tetramer- CD8+ T cells. **E)** The ratio of tetramer+ CD8+ T cells to Tregs. One (B) or two (C-E) way ANOVAs were performed, and significance is reported as follows: * = p<0.05; **** = p<0.0001

WTX-124 Activates Tumor Specific CD8+ T Cells

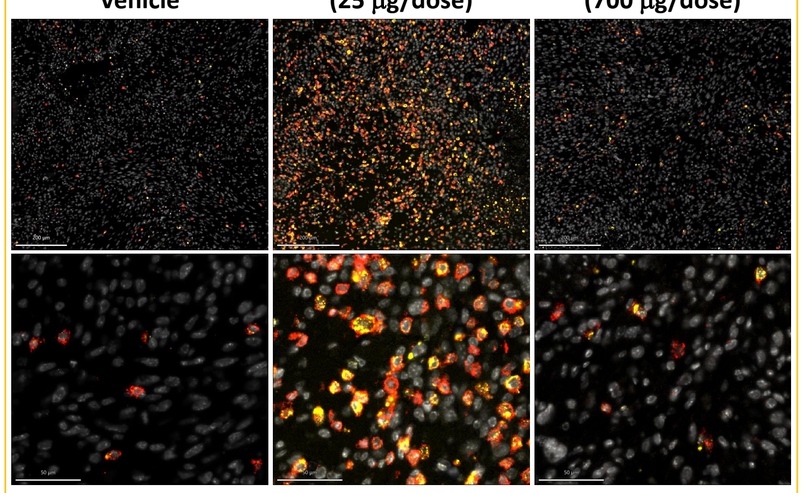
Wildtype IL-2 is Required to Generate Polyfunctional Tumor Specific CD8 T Cells



MC38 tumor bearing mice were dosed as previously described. Doses are specified in the figure. Tumors were harvested on Day 5 or Day 11 for analysis. Tumors were dissociated and analyzed by flow cytometry after *ex vivo* restimulation for cytokine staining at the specified timepoints. **A)** Representative flow cytometry plots and **B)** quantification. Statistics were generated using a two-way ANOVA, and significance is reported as follows: * = p<0.05; **** = p<0.0001

WTX-124 Drives Granzyme B Production in the TME

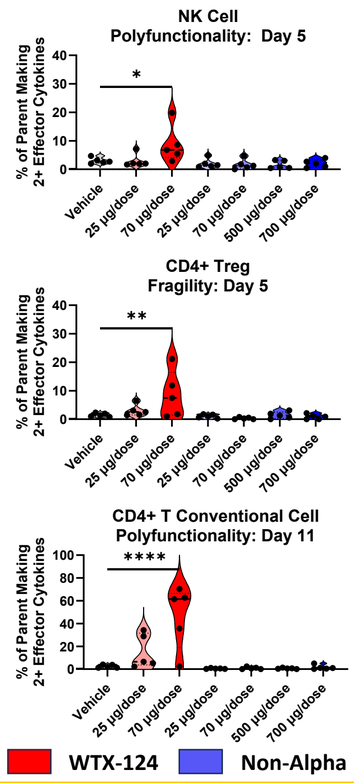
Wildtype IL-2 Robustly Induces Effector Cytokine Production



MC38 tumor bearing mice were dosed as previously described. Doses are specified in the figure. Tumors were harvested on Day 5 for analysis. Multiplexed immunofluorescence analysis was performed on FFPE tumors, with representative images shown.

WTX-124 Activates Additional Effector Cells

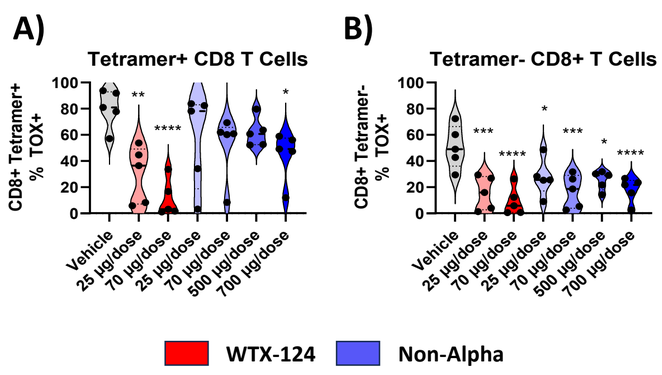
Wildtype IL-2 is Required for Immune Cell Activation



MC38 tumor bearing mice were dosed as previously described. Doses are specified in the figure. Tumors were harvested at the stated timepoints, dissociated, and analyzed by flow cytometry after *ex vivo* restimulation for cytokine staining at the specified timepoints. Quantification of effector cytokine production by NK cells, CD4+ Tregs, or CD4+ T conventional cells is shown. Statistics were generated using a two-way ANOVA, and significance is reported as follows: ** = p<0.01; **** = p<0.0001

IL-2 Protects CD8+ T Cells from Exhaustion

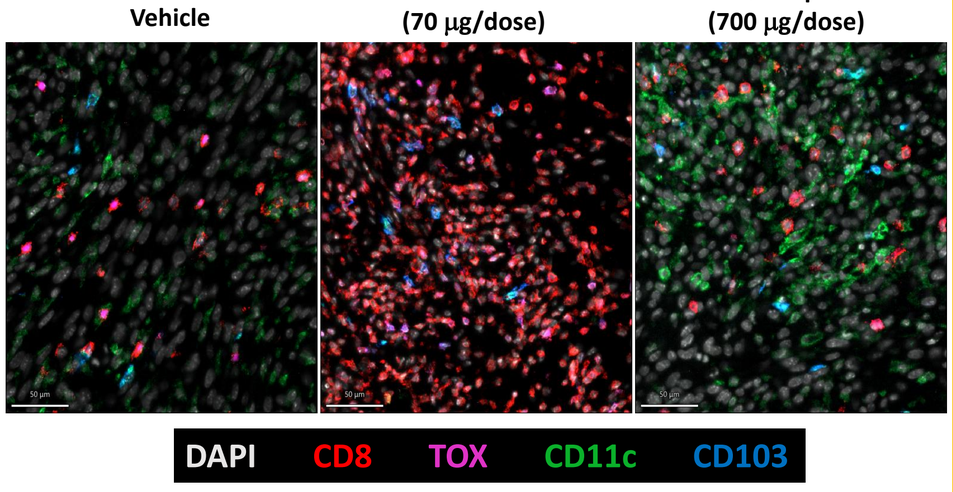
WTX-124 Strongly Protects Tumor Specific T Cells



MC38 tumor bearing mice were dosed as previously described. Doses are specified in the figure. Tumors were harvested at Day 5 for analysis. Tumors were dissociated and analyzed by flow cytometry. Frequency of **A)** tetramer+ or **B)** tetramer- CD8+ T cells expressing TOX, a transcription factor associated with T cell exhaustion. Statistics were generated using a two-way ANOVA, and significance is reported as follows: * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001

WTX-124 Drives Clustering of CD8+ T Cells with CD103+ DCs

Suggestive of Ongoing T cell Activation Within the TME



MC38 tumor bearing mice were dosed as previously described. Tumors were harvested on Day 5 for analysis. Multiplexed immunofluorescence analysis was performed on FFPE tumors, with the various markers examined specified in the figure.

CONCLUSIONS and REFERENCES

- Wildtype IL-2 was substantially more active than a Non-Alpha IL-2 variant when tested on activated T cells, due to induction of high affinity receptor (CD25/CD122/CD132) for IL-2.
- WTX-124, an INDUKINE molecule containing wildtype IL-2, generated robust anti-tumor activity in the MC38 model and promoted the expansion and activation of tumor specific CD8+ T cells.
- Meanwhile, a variant INDUKINE molecule containing Non-Alpha IL-2 failed to generate anti-tumor activity, to drive tumor specific CD8+ T cell expansion, or to activate tumor infiltrating immune cells even when dosed up to 28X higher than the active dose of WTX-124.
- While both INDUKINE molecules protected tumor infiltrating CD8+ T cells from exhaustion, only WTX-124 was able to induce an effector phenotype in tumor specific CD8+ T cells.
- Only treatment with WTX-124 resulted in clustering of CD8+ T cells with CD103+ cross presenting dendritic cells within the tumor.

1. Klein, C. et al. Cergutuzumab amunaleukin (CEA-IL2v), a CEA-targeted IL-2 variant-based immunocytokine for combination cancer immunotherapy: Overcoming limitations of aldesleukin and conventional IL-2-based immunocytokines. Oncoimmunology 6, (2017).
2. Wu, W., Chia, T., Lu, J. et al. IL-2Rα-biased agonist enhances antitumor immunity by invigorating tumor-infiltrating CD25+CD8+ T cells. Nat Cancer (2023). https://doi.org/10.1038/s43018-023-00612-0
3. Subramanian, K. et al. PK/RO Modeling of WTX-124, a Tumor-Activated IL-2 Prodrug, Highlights the Potential for a Substantially Improved Therapeutic Index Compared to Other IL-2 Molecules. Abstract #1074 SITC 2023
4. Nirschl, C. J. et al. Discovery of a Conditionally Activated IL-2 that Promotes Antitumor Immunity and Induces Tumor Regression. Cancer Immunol. Res. 10, 581–596 (2022).

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